THE NAUTILUS 125(2):63–71, 2011 Page 63

DNA barcoding reveals *Brachidontes* (Bivalvia: Mytilidae) from two ecologically distinct intertidal habitats on Long Key, Florida Keys, are cryptic species, not ecotypes

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ABSTRACT

The nominal morphospecies *Brachidontes exustus* (Linnaeus, 1758) represents a cryptic species complex with multiple genetic disjunctions resulting in regionally dominant, but range-restricted, species throughout the western Atlantic, Caribbean, and Gulf of Mexico. In the Florida Keys, four species were previously identified using molecular techniques. Specimens were collected in January 2005 from two distinct habitats, a seawall and a mangrove, on Long Key, Florida Keys. The locations are separated by <5 km. Eight specimens from the mangrove and four from the seawall were sequenced for the mitochondrial COI gene. Two seawall specimens were sequenced at the internal transcribed spacer 2 (ITS-2), in the nuclear ribosomal gene cluster, after the COI sequences appeared to be from the male mitochondrial line. The COI and ITS-2 sequences indicate that the two locations on Long Key, Florida Keys, have different single-species populations. The four seawall specimens were the Antillean species while the eight mangrove specimens were the Gulf species. Given that these mussels broadcast spawn, with subsequent planktotrophic larval development, the sites likely share a common pool of potential larval recruits. Single-species populations at each location are suggestive of habitat partitioning, ecological filters, or differential recruitment.

Additional keywords: Brachidontes, cryptic species, Florida Keys, cytochrome *c* oxidase subunit I (COI), DNA barcoding

INTRODUCTION

The scorched mussel, *Brachidontes exustus* (Linnaeus, 1758), is a small bivalve (maximum length = 25 mm) in the family Mytilidae that commonly inhabits rock pilings, seawalls, and wharf pilings in the intertidal zone and is most abundant in the lower intertidal (Seed, 1980). Abbott (1974) considered *B. exustus* and *Brachidontes domingensis* (Lamarck, 1819) separate species with the range of *B. exustus* from North Carolina to Texas and the West Indies and *B. domingensis* from Bermuda, the Bahamas, and southeast Florida and throughout the Caribbean. Later authors (Rios, 1985; Jensen and Harasewych, 1986) introduced taxonomic confusion by reciprocally synonymizing the two names. Rios (1985), working in Brazil, considered *B. exustus* the primary name and *B. domingensis* a synonym. Jensen and Harasewych (1986), publishing in a volume on the fauna of Bermuda, used the more recent *B. domingensis* as the primary name. Although these authors were working at opposite ends of the range and designated a different primary name, the combined conclusions suggested that a single intertidal *Brachidontes* species spans the entire range from North Carolina to Argentina.

This morphospecies has been shown to be a cryptic species complex throughout its western Atlantic, Gulf of Mexico, and Caribbean Basin range. Five molecular taxonomic units (MTU) within the nominal species have been identified using nuclear and mitochondrial gene trees. Each MTU has been given an informal name corresponding to its core geographical distribution; Antilles, Atlantic, Bahamas, Gulf, and Western Caribbean (Lee and Ó Foighil, 2005). Four of these cryptic species (Antilles, Atlantic, Bahamas, and Gulf) have been found in the Florida Keys (Lee and Ó Foighil, 2004). The four species are nested, two sister-species apiece, within two of the three main branches revealed by a ribosomal 28S gene tree (Lee and Ó Foighil, 2005). The five cryptic species have not yet been formally described and currently remain under the single morphospecies taxon, *Brachidontes exustus*.

DNA barcodes have been proposed as a method for genetically cataloging the world's biological diversity

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(Hebert et al., 2003). The proposed "barcode" is a short section of the mitochondrial genome that can identify unknown specimens when compared to an existing database of sequences, and are particularly useful in identifying cryptic species (Hebert et al., 2004; Gomez et al., 2007) or other closely-related species (Packer et al., 2009). The 5′ region of the cytochrome *c* oxidase subunit I (COI) mitochondrial gene, amplified by the well-known "universal" COI primer pair of Folmer et al. (1994), has emerged as the agreed-upon sequence for DNA barcoding for most species. Some bivalves have a unique mitochondrial inheritance system that may complicate the effectiveness of DNA barcoding. Doubly uniparental inheritance (DUI) of sex-linked mitochondrial lineages (Zouros et al., 1994; Mizi et al., 2005) results in maternally and paternally-inherited mitochondrial genomes coexisting in males. DNA barcodes that do not match the known sequences of the species may result if tissues used for DNA extraction are enriched with the male line of mitochondria.

Lee and Ó Foighil (2004) collected nominal *Brachidontes exustus* from three rocky intertidal sites from the Florida Keys (Figure 1). The Bahamian and Gulf species were most commonly encountered. The Bahamian species dominated their most southerly collection site at Boca Chica Key, while the Gulf species was most common at the Horseshoe site on Spanish Harbor/West Summerland Key, though intermingled with individuals of the Bahamian species. The Atlantic and Antillean species were much less common and were identified from very few individuals. The only genetically verified records of these two presumably rarer species include a single Atlantic species specimen found with an otherwise Bahamian population at Boca Chica Key, and three sub-adults of the Antillean species found along with five Bahamian species individuals at Key Biscayne (Lee and Ó Foighil, 2004). Lee and Ó Foighil (2004) did not collect specimens from mangroves, a habitat for *B. exustus* encountered by Bennett and Willan (2003) in the Florida Keys, and subsequently by Lee and Ó Foighil (2005), where they found the Western Caribbean and Atlantic species in a mixed population.

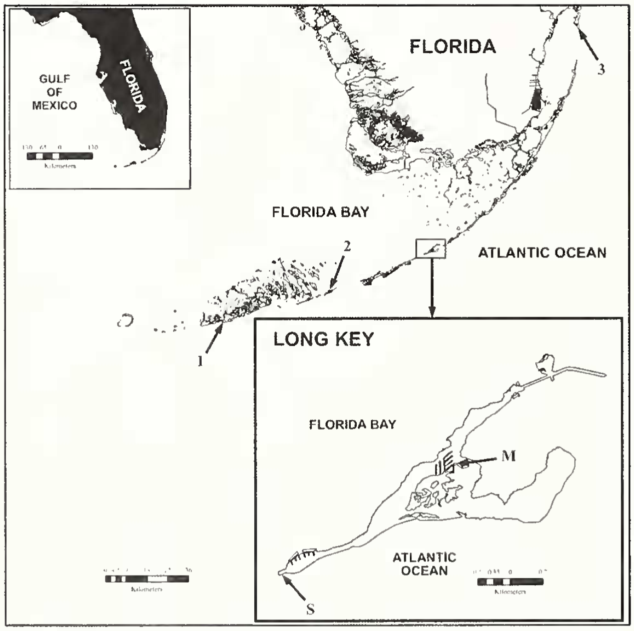


Figure 1. Map of the Florida Keys showing the collection locations of Lee and Ó Foighil (2004). 1 = Boca Chica Key, 2 = The horseshoe site on Spanish Harbor/West Summerland Key, and 3 = Key Biscayne. Upper left inset showing the Floridian peninsula. Lower left inset showing Long Key and collection locations of this study. S = seawall habitat location and M = mangrove habitat location.

Sibling species and cryptic species complexes in what were previously believed to be single species are well known for many marine taxa (Knowlton, 1993), including bivalves (Marko and Moran, 2009). In the *Mytilus* complex of *M. californianus*, *M. galloprovincialis*, and *M. trossulus* on the western coast of North America, the distributions appear to be maintained by subtle habitat differences that influence local post-recruitment dominance (Heath et al., 1995; Johnson and Geller, 2006). Likewise, local habitat differences may influence *Brachidontes* cryptic species distributions in the Florida Keys. The goal of the present study was to determine the species distribution in the two distinct habitat types on Long Key, where *B. exustus* was encountered. A previous study of Floridian *Brachidontes* spp. (Lee and Ó Foighil, 2004) did not include specimens from seawall or mangrove habitats, nor were multiple samples taken from different habitat locations on a single island.

MATERIALS AND METHODS

Study Area

Long Key, a 6-km long Y-shaped island in approximately the middle of the Florida Keys archipelago, has an interior lagoon fringed by mangroves with a connection to the bayside of the island (Figure 1). The nearest mile marker on US Route 1 serves as a useful universal landmark for the Florida Keys and the number of the nearest mile marker is included with the collection locations. Mile marker 0 corresponds to the western edge of Key West, and the mile number increases as one moves east and north towards mainland Florida.

The first collection location (latitude 24°48.10 N, longitude 80°50.58 W) is an ocean-side vertical concrete seawall near mile marker 66 at the west end of Long Key, adjacent to the Long Key Viaduct (Figure 1). The lowest intertidal zone of the seawall is approximately 1 m above a sandy bottom. Wave exposure at this location is high and directly impacts the exposed substrate. The second collection location (latitude 24°49.22 N, longitude 80°48.45 W), near mile marker 68 and approximately 4 km to the northeast, is a mangrove-lined boating channel that leads from the bayside of the island to the interior lagoon and is protected from direct wave action.

SAMPLE COLLECTION

Living specimens were collected in January 2005. Four small individuals approximately 11 mm in length were found in the lower intertidal on the seawall and all were collected. A total of 46 individuals ranging in size from 6 mm to 22 mm were collected from an abundant population in the mangrove habitat. All specimens were immediately transferred to a seawater flow-through system and maintained alive for several days before being frozen at −40°C. The samples were subsequently transported on dry ice and stored at −80°C prior to dissection and molecular characterization.

Specimens were thawed to room temperature, dissected, and the soft tissues separated from the shell. The disarticulated left valve of each subsequently typed specimen was photographed using a Nikon digital SLR camera mounted on a Zeiss Stemi 2000-C dissecting scope.

DNA EXTRACTION AND MOLECULAR CHARACTERIZATION

Total genomic DNA was isolated from approximately 20 mg of tissue from either the posterior adductor muscle in larger specimens or mantle tissue in smaller specimens. The extraction was accomplished with a DNeasy Tissue Kit (Qiagen, Valencia, California) according to the manufacturer's instructions. Extracts were stored at −20°C until used as PCR templates.

The target fragment for DNA barcodes, the 5′ end of the cytochrome *c* oxidase subunit I (COI) gene from the mitochondrial genome, was amplified by polymerase chain reactions (PCR) using Taq PCR Mastermix (Qiagen, Valencia, California) and the well known "universal" COI primer pair, LOC 1490 and HOC 2198, from Folmer et al. (1994) with a thermal cycler protocol of 3 min initial denaturing at 95°C followed by 35 cycles of 95°C for 1 min, 40°C for 1 min, 72°C for 1.5 min, with a final 7 min extension at 72°C.

A second target fragment, the second internal transcribed spacer (ITS2) region from the nuclear ribosomal gene cluster, was sequenced for two specimens from the seawall for reasons discussed below. The ITS-2 was amplified with the forward primer (5′-CATCGATATCTTGAACGC-3′) from Lopez-Piñión et al. (2002) initially designed for European scallops and the reverse primer (5′-GCTCTTCCCGCTTCACTCG-3′) from Xu et al. (2001) initially designed for various species of *Crassostrea*. The thermal cycler protocol was 3 min initial denaturing at 94°C followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s with a final extension of 5 min at 72°C. All reactions were prepared in 50-µl volumes and a negative control containing all reagents and the primer pair, without the DNA template, was included with each amplification series. The resulting PCR products, controls, and a 100-bp ladder were run on a 2% agarose gel, stained with ethidium bromide, and photographed under UV transillumination.

PCR products were checked for the appropriate size, purified with a MinElute kit (Qiagen, Valencia, California) according to the manufacturer's directions and subsequently directly cycle-sequenced in both directions with the above amplification primers utilizing the BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Forester City, California) and an automated DNA sequencer (310 Avant Genetic Analyzer, Applied Biosystems, Forester City, California).

Chromatograms of sequences were edited manually by comparing both strands in 4Peaks (by Griekspoor and Groothuis, Mekentosj.com). The edited sequences were compared to an existing database of sequences using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) of the National Center for Biotechnology Information (NCBI). The most significant alignment with the highest percent congruence to published sequences was treated as the species identification.

GENE TREE

A subsample of COI sequences representing each of the five cryptic species and their within-species diversity from their total geographic range were obtained from GenBank (accession numbers AY621879, AY621909, AY621911, AY621913, AY621914, AY825105, AY825201, AY825202, AY825204 and AY825216) (Lee and Ó Foighil, 2004; Lee and Ó Foighil, 2005) and combined with nine of the ten newly generated maternal COI sequences. The sequences were aligned in Clustal X and a gene tree was created using the neighbor-joining method.

RESULTS

SPECIES ASSIGNMENT FROM SEQUENCES

The gene tree created from the seven mangrove and two seawall specimen COI sequences and select COI sequences obtained from GenBank show the relationships of the specimens to the five cryptic species within the complex (Figure 2). Two specimens from the seawall location were excluded as explained below. Table 1 shows species identifications based on comparisons to the NCBI database for all sequences generated in this study.

SEAWALL LOCATION

The resulting COI sequences, when compared to the NCBI database, had closest matches to an Antillean species sequence and a Biscayne clade species sequence. The Biscayne clade was later named the Antilles species after gene trees from the full western Atlantic and Caribbean range showed that some specimens initially collected from Key Biscayne during the sampling of Lee and Ó Foighil (2004) were related to the dominant species of the Antillean archipelago (Lee and Ó Foighil, 2005). The match to an Antillean COI sequence was 100% for two of the four specimens (Lee and Ó Foighil, 2004; Lee and Ó Foighil, 2005). The two other sequences were hard to clean and edit because of many instances of unclear peaks in both the

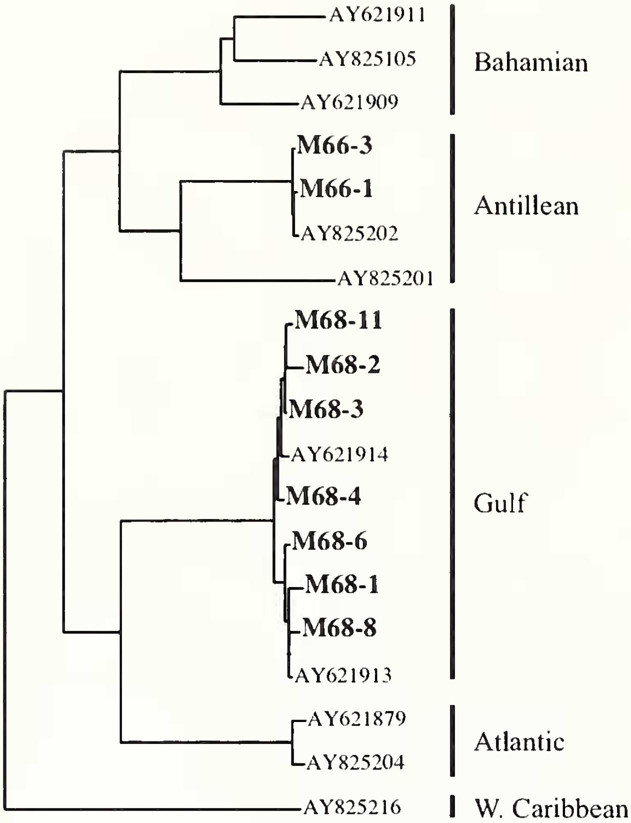


Figure 2. Phylogram generated from DNA sequences of the COI gene from representatives of cryptic species within the *Brachidontes exustus* morphospeices complex. GenBank sequence numbers and corresponding cryptic species are labeled. The mile location and specimen number labeling the sequences generated in this study are in bold.

forward and reverse sequences. The two cleaned and edited sequences had sections at both ends that were ambiguous, which were cropped from the full sequence before being compared to the NCBI database. The two sequences were 543 and 621 nucleotides long and returned respectively an 82% and 83% closest match to GenBank accession number AY621945, a male mitochondrial line COI sequence of a Bahamian species specimen collected at Boca Chica Key (Lee and Ó Foighil, 2004). The most congruent non-male COI sequence match for both specimens was an Antillean species sequence–AY825208 (Lee and Ó Foighil, 2005), with 80% congruence.

The specimens that yielded the presumptive male COI sequence were subsequently sequenced at the ITS-2 locus to determine their species identity. The resulting sequences were 355 nucleotides long and when compared to the NCBI database returned either 100% or 99% congruence with AY621970. Sequence AY621970 is the complete sequence of the internal transcribed spacer 2, and flanking portions of the 5.8S and 28S ribosomal RNA genes, obtained from voucher specimen 300123.6 of the University of Michigan Museum of Zoology. The specimen was collected from Key Biscayne, Florida (Lee and Ó Foighil, 2004). Both of the newly-generated ITS2 sequences contained a 4-nucleotide deletion relative to the Bahamian sequences at aligned location 130, a polymorphism consistent with the single Antillean species ITS2 sequence in GenBank, and lacking in any Bahamian species ITS2 sequence. The four seawall specimens are thus grouped with the Antillean species, by either mitochondrial COI or nuclear ITS-2 sequences. The Antillean species is one of the two less common species encountered by Lee and Ó Foighil (2004) in the Florida Keys and this new record more than doubles the confirmed number of individuals of this species collected in South Florida.

MANGROVE LOCATION

Eight specimens from the mangrove location were chosen for sequencing based on extremes in size and shape revealed by a Principal Components Analysis based on shell morphometrics (unpublished data). The expectation is that if more than one species is present at this location the extremes of size and shape would likely include representatives of the cryptic species. COI

Table 1. Specimen, habitat type, sequenced gene, fragment length in nucleotides used for database comparisons, NCBI accession number of sequence with most significant alignment, percentage congruence of *de novo* sequence to NCBI sequence, and resulting species identification of specimen based on closest matching sequence.

Specimen	Habitat	Gene	Length	Significant alignment	Congruence	Species
M66-1	seawall	COI	660	AY825154	100%	Antilles
M66-2	seawall	ITS-2	355	AY621970	99%	Antilles
M66-3	seawall	COI	660	AY621849	99%	Antilles
M66-4	seawall	ITS-2	355	AY621970	99%	Antilles
M68-1	mangrove	COI	660	AY621885	99%	Gulf
M68-2	mangrove	COI	660	AY621915	99%	Gulf
M68-3	mangrove	COI	660	AY621915	100%	Gulf
M68-4	mangrove	COI	660	AY621897	99%	Gulf
M68-5	mangrove	COI	474	AY621915	99%	Gulf
M68-6	mangrove	COI	660	AY621913	99%	Gulf
M68-8	mangrove	COI	660	AY621913	99%	Gulf
M68-11	mangrove	COI	660	AY621915	99%	Gulf

sequences of seven specimens were 660 nucleotides long and when compared to the NCBI database returned in all cases a Gulf species closest match with 100% or 99% congruence (Table 1). The COI sequence of one individual, M68-5, had many ambiguous peaks at both the 5′ and 3′ ends and a clean sequence of 474 nucleotides was excised from the longer sequence. This 474-nucleotide-long fragment was compared to the NCBI database and returned 99% congruence to a Gulf species COI sequence. The shorter COI sequence was not included in the gene tree, but nonetheless serves as a positive identification of this specimen as the Gulf species.

DISCUSSION

One major criticism of DNA barcodes as a method of species designation is the possibility of misidentifications based on errors in the database of linked sequences. This is a problem with using GenBank data, where a submitter of the sequence names the taxon to which the sequence is linked. There is no outside review of the validity of the taxonomy and only the original submitter can change the name attached to the sequence. *B. exustus* appears to suffer from just such a problem. The linked taxon in the NCBI database is *Hormomya exustus*, a generic name long ago synonymized to *Brachidontes exustus* (Soot-Ryen, 1969). This problem in taxonomy now extends to the Barcode of Life Database (BOLD) by virtue of its linking to the NCBI database. BOLD utilizes only a standardized 648-nucleotide fragment from voucher specimens whose origin and current status are recorded (Hebert and Gregory, 2005). Additionally, without the proper taxonomic treatment of the cryptic species, there is no way to know that cryptic species are involved without a detailed knowledge of the clade information included for each *Brachidontes* specimen in the GenBank database. While the BOLD attempts to be more rigorous than the GenBank database because voucher specimens are required for each sequence, the lack of taxonomic treatment for each of the cryptic species within the *Brachidontes exustus* complex likely dooms them to obscurity until a proper taxonomic treatment is undertaken (Schlick-Steiner et al., 2007).

Lee and Ó Foighil (2005), remarking on the distributions of the five *Brachidontes* species throughout the entire known Atlantic, Gulf of Mexico, Bahamas, and Caribbean range, note that the Gulf/Atlantic clade appears mostly on continental margins, while the Bahamas/Antilles clade is found predominately on oceanic islands. The abiotic aspects of the mangrove habitat may be more similar to continental margins, while the seawall habitat may be more similar to the conditions found on oceanic islands. Distinct differences between the bivalve species assemblages on the bay and ocean-sides of the Florida Keys archipelago are known (Bieler and Mikkelsen, 2004; Mikkelsen and Bieler, 2007) and the distribution of *Brachidontes* spp. on Long Key are likely representative of the same environmental factors that influence other bivalve species' distributions. Furthermore, the environmental conditions of the core distributions of the individual *Brachidontes* species in the western Atlantic, Gulf of Mexico, Bahamas, and Caribbean Sea observed by Lee and Ó Foighil (2005) relate to the conditions coinciding with the species' distribution on Long Key. High salinity and low productivity associated with ocean islands are present at the seawall, while lower salinity and high productivity conditions associated with continental margins are present at the mangrove site.

Only four individuals were found on the approximately 250 m of seawall after considerable effort and all individuals were collected and used for genetic typing. Based on the very low abundance and small size of the collected specimens, this location likely represents a marginal habitat for *Brachidontes* spp., survivable by only one of the species within the complex. The four seawall individuals were only half the maximum length of individuals found in the mangrove habitat yet they are likely sexually mature. Mytilids have doubly uniparental inheritance of sex-linked mitochondrial lineages (DUI) (Zouros et al., 1994) and the initial extraction appears to have been contaminated by sperm from a portion of gonadal tissue extracted with the mantle tissue. Only sexually mature males would have sperm in sufficient quantities to make a significant contribution of the paternally-inherited COI sequence to the PCR amplification. The smallest reproductive individuals encountered during a study of the gametogenic cycle of *B. exustus* (presumably the Atlantic species) at Wassaw Island, Georgia were about 10 mm long (Sweeney and Walker, 1998). This is slightly smaller than the specimens collected from the seawall on Long Key. If the threshold for reproductive size is similar for the Atlantic and Antillean species, then the seawall specimens are within the known limits of size for reproductive individuals.

The presumptive male sequences are slightly less congruent with the Antillean species maternal COI line (80%) than they are with the known male COI sequences (82%) from Bahamian specimens. An alignment of the two new presumptive male sequence fragments in Clustal X showed the sequences to be very close to each other with few single nucleotide substitutions and one section of over 100 nucleotides identical between the two sequences. The close congruence suggests that the sequences are not amalgamations of amplified maternal and paternal COI PCR products but are the sequences for the male COI of the Antillean species. In other bivalves that display DUI the male mitochondrial genome has been hypothesized to have been replaced and reset to the female mitochondrial line (Mizi et al., 2005). The similar 20% differences between the Bahamian male COI and the maternal Antillean species COI may be a result of a resetting of the male COI line to the maternal mitochondrial line after speciation. A fuller gene tree comprising the male COI sequences of each species within the complex would answer the questions of male mitochondrial genome origins. Contamination by mitochondrial DNA from sperm would not affect the amplification of the nuclear ITS-2 used for the species designation of the two specimens that yielded a presumptive male COI sequence.

Ideally, a range of size classes from the seawall location would have been used for species identifications, but all four of the specimens found on the seawall were approximately 11 mm in length. Wave exposure at the seawall is a prominent environmental feature and could be limiting abundance and survival at the seawall. At Moss Landing, California, and along the central coast of California, *Mytilus* spp. occurs in mixed populations in which the dominant species differs among age classes. When wave exposure is more intense, one species of *Mytilus* comprises a greater proportion of adults than juveniles, suggesting that the adults of this one species are more resistant to dislodgement by wave action (Heath et al., 1996; Johnson and Geller, 2006). Wave exposure may play a role in the species composition at the seawall location, although wave exposure alone cannot explain the observed pattern in the mangrove habitat. Antillean species individuals should be present in the mangrove, a habitat with low wave exposure, if wave-induced dislodgement were the only environmental attribute influencing the distribution.

The harsh conditions under high wave exposure may limit the growth or set a maximum attainable size for the few individuals that persist at the seawall. Alternatively, larger individuals may still be susceptible to dislodgement once over a certain size threshold. While all of the seawall specimens were about the same length, they may be of varying ages and not representative of a single settlement cohort because growth may slow to near zero under the stress of intense wave exposure.

Unfortunately, the small sample size at the seawall location and limited number of collection locations from Long Key truncate the explanatory power of this study. The inclusion of the apparently marginal habitat at the seawall for a study of species distributions in the Florida Keys became a necessity because many locations that seemed suitable for *Brachidontes* spp. did not have populations and specimens were collected whenever they were encountered. Nonetheless, the stark contrast of species-specific populations of cryptic species at such proximal locations and differing habitats warranted including such a marginal habitat for comparison. The only other record of the Antillean species in the Florida Keys is from a habitat at Key Biscayne with very low abundances of *Brachidontes* spp. (Lee and Ó Foighil, 2004). The Antillean species may be more common in the Florida Keys than the records to date suggest because marginal habitats have not been actively searched in the few existing studies on this species complex.

At the mangrove location, specimens were found as dense clusters of individuals within the byssal threads of another bivalve, *Isognomon* sp., which were, in turn, attached to the mangrove roots. It was not uncommon to find 20 or more individuals within the threads of a single *Isognomon* sp. Specimens were collected from the same mangrove location during the summer of 2002 but were not used in this study. Shell morphology over the 2.5-year period remained consistent and the largest shells from the two collection times were about the same length (≈ 25 mm). Unlike the individuals collected from the seawall, the mangrove specimens identified by COI sequences spanned the entire range of sizes collected and the specimens chosen for sequencing were at the extremes of size and shape variation. If individual settlement events were dominated by a single species, there should be a change in the species composition at the various size classes and between the two collections times. Based on the consistent species designation by sequence data over the entire range of size classes, combined with the consistent shell morphology, it appears that the exclusivity of the Gulf species at the mangrove location was maintained over the 2.5 years between collections. This would be unlikely if the observed distribution were the result of stochastic larval settlement.

In contrast to the Long Key locations, other sites in the Florida Keys have more than one cryptic species of *Brachidontes exustus* coexisting on the same substrate (Lee and Ó Foighil, 2004). Neither of the Long Key collection locations was the rocky shore habitat sampled by Lee and Ó Foighil (2004). Mixed-species populations may reflect an overlap of suitable habitat that only exists at some rocky shore locations. Species may be contemporaneous on the same rocky shore by habitat partitioning, by depth, for example. Abiotic factors or biotic interactions could limit one or both species distribution on micro-scales in such a way to facilitate coexistence. Further investigation of the species' distributions, at spatial scales of individuals to islands, is warranted to elucidate the mechanisms of coexistence or exclusion among the species within the complex.

Differential larval transport from single-clade source populations could potentially impact adult distributions. If the larvae of each species are not transported to all potential locations, the differences noted in the species distributions could be an artifact of larval transport processes and not ecological filtering. This seems unlikely given that *Brachidontes exustus* from the North American Atlantic coast, as well as the closely related species *B. granulata*, *B. modiolus*, and *B. variabilis*, broadcast spawn with planktotrophic larvae that can stay in the water column for up to 40 days before settlement (Campos and Ramorino, 1980; Fields and Moore, 1983; Morton, 1988; Fuller and Lutz, 1989). The planktonic larval phase would provide ample opportunity for tidal fluxes and currents to overwhelm any local larval source cohesiveness.

Studies of connectivity using biophysical modeling and elemental tracking suggest that long-range dispersal may be much rarer than simple assumptions of pelagic larval duration and ocean currents would indicate (Cowen et al., 2006; Becker et al., 2007). The four kilometers between the Long Key collection locations are well within the ecologically relevant magnitudes of dispersal of 10 to 100 km of the Cowen et al. (2006) model and the 20 to 30 km suggested by experimental approach of Becker et al. (2007). Within the dispersal distances advanced by these researchers, larvae from both Long Key populations would have a very high likelihood of being delivered to the nearby location. Additionally, their results imply that larvae of all *Brachidontes* spp. present in the Florida Keys would be delivered to all suitable Florida Keys locations, suggesting that species-specific populations must reflect recruitment or post-recruitment processes and not clade-limited settlement events.

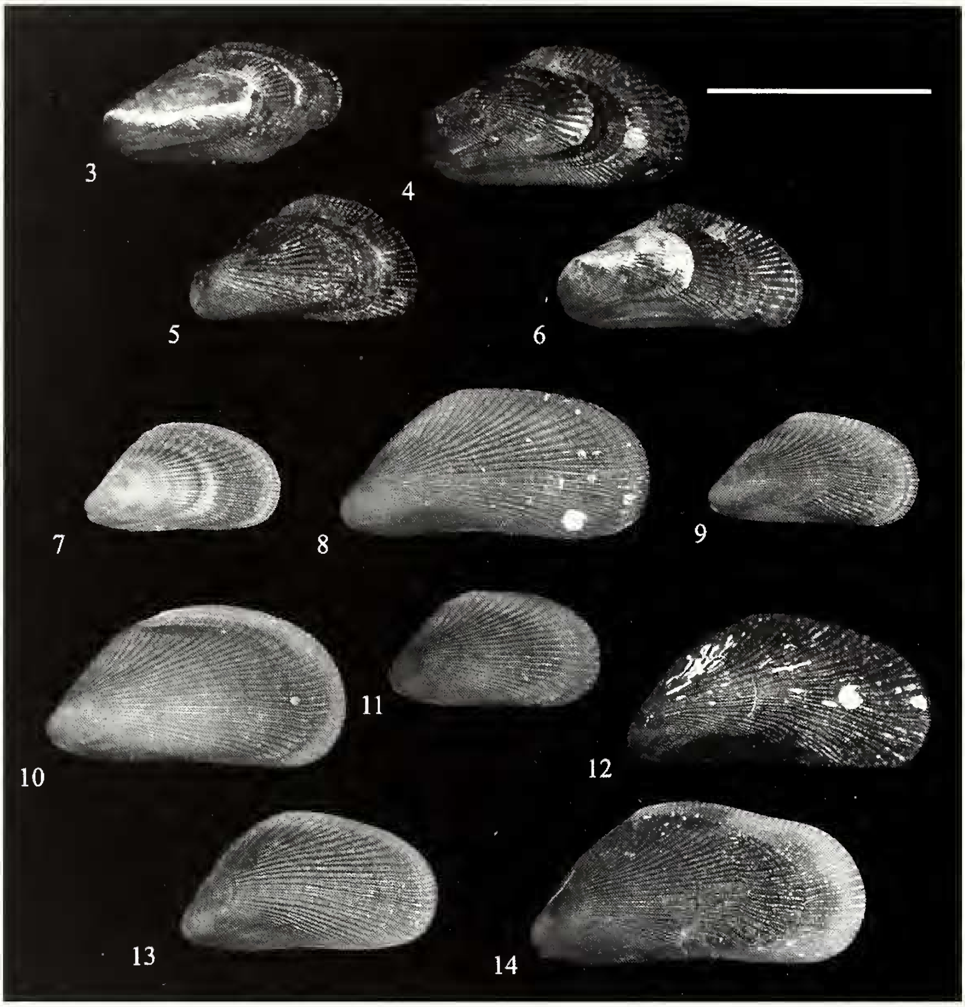
Previous authors (Lee and Ó Foighil, 2005) were unable to determine the species of a given individual by morphology alone. However, representative shell shapes are included in their nuclear and mitochondrial gene trees. A previous investigation of plasticity of *Brachidontes* spp. in the Florida Keys found large overlaps in the shell morphologies of specimens sampled from habitats with different wave exposures (Bennett and Wilan, 2003). Their conclusion of environmentally-induced plasticity is severely undermined by the discovery of the cryptic species complex in the Florida Keys. Two of Bennett and Wilan's collection locations, the Horseshoe site on Spanish Harbor/West Summerland Key and the mangrove on Long Key, do not have the same species distributions. The Horseshoe site has a mixture of Gulf and Bahamian species (Lee and Ó Foighil, 2004), while the mangrove location population is shown here to be limited to the Gulf species. The broad morphological variation that was observed at the sites sampled by Bennett and Wilan (2003) is probably the result of morphological differences between the cryptic species with the overlap in shell morphology between locations being the result of conserved Gulf species morphology. Regardless, the morphological variation observed does not reflect ecotypic differences within a single species.

Notwithstanding Lee and Ó Foighil's (2005) observations on morphology, there were some obvious qualitative differences in gross shell morphology between the specimens from the two Long Key locations. Shells collected from the seawall were thicker with more robust ribbing and were wider across both valves compared to shells of similar length collected from the mangrove habitat. When viewed laterally, the overall shell outline of the seawall specimens was more modioliform (Figures 3 through 6), while the mangrove specimens were more mytiliform (Figures 7 through 14). For the seawall specimens, the anterior margin was more rounded, the umbo in a more dorsal orientation, and the overall ventral-dorsal margin angle less than in specimens of similar size from the mangrove. These morphological differences match the known phenotypic plasticity of mytilids in habitats with different wave exposures (Seed, 1968), salinities (Nalesso et al., 1992), or shorelines (Morton, 1991). However, in light of the existence of cryptic species, it is unclear whether the observed morphological variation arises from phenotypic expression, genotypic constraints, or a combination of the two.

Multivariate statistical methods to determine species by shell morphometrics may be constructed with a large number of specimens that are unequivocally assigned to species by molecular methods. However, a statistical method is only possible if there are consistent genotype-dependent morphological differences that overwhelm any phenotypically-variable morphology. Sampling individuals from locations where the species coexist is the obvious starting point for examining this question because mussels from these locations should presumably be exposed to identical phenotype-inducing environmental cues and would be expected to display similar phenotypic responses. Any consistent morphological differences between the species at locations of coexistence would only be those whose expression is more dependent on genotype than phenotype. Morphological discrimination of cryptic species would make manipulation experiments possible because the mussels would not have to be killed in order to determine species. Reciprocal transplant or common-brood experiments could then be used to investigate the precise mechanisms regulating species survivability at single and multiple-species locations.

The discovery of cryptic species in such close proximity in the Florida Keys opens the possibility that hidden biodiversity within *Brachidontes* spp. populations may be found in other locations throughout the Caribbean if sampling efforts were increased. Most of the Caribbean island locations sampled by Lee and Ó Foighil (2005) were from a single collection site and yielded a single species. The two locations sampled on Trinidad, which yielded different species, provide an exception. While the two Trinidadian locations are about 20 km apart, the Atlantic species was collected at the town of Chaguaramas and the Antillean species was collected at Maracas Bay. Although the habitat types are not listed, habitat specificity similar to what was observed on Long Key may exist at these Trinidadian collection locations. Maracas Bay is on the north side of the island with an exposure to the Atlantic Ocean, while Chaguaramas is to the west, on the Gulf of Paria. The species distributions on Trinidad may be influenced by habitats with oceanic island and continental margin-like environments, as with the distributions observed at Long Key.

Many intriguing questions remain regarding the ecological mechanisms that affect the distribution of this cryptic species complex in the Florida Keys and the western Atlantic. More records of the species in close proximity may be discovered if sampling efforts are increased. Mangrove habitats in the Caribbean may support populations that are not the recognized dominant regional species. Understanding the mechanisms in specific habitats that maintain or exclude each species within the complex offers the potential to ask questions regarding interactions of very closely related species in the oceans. This little-studied complex may become a good model to investigate these types of difficult to answer questions.



Figures 3–14. Disarticulated left valves of 12 specimens from two sample locations on Long Key. Figure numbers of each shell are at the umbo. Shells in Figures 3 to 6 were collected from the seawall habitat, M66, and are the Antillean species. Shells in Figures 7 to 14 were collected from the mangrove habitat, M68, and are the Gulf species. Specimen codes for each figure are: 3) M66-1; 4) M66-2; 5) M66-3; 6) M66-4; 7) M68-1; 8) M68-2; 9) M68-3; 10) M68-4; 11) M68-5; 12) M68-6; 13) M68-8; 14) M68-11. Scale bar = 10 mm.

ACKNOWLEDGMENTS

Constantino Vetriani was instrumental in the execution of the laboratory portion of this project along with students in the Deep-Sea Microbiology Lab at Rutgers University. Special thanks to Rüdiger Bieler and Paula Mikkelsen, co-organizers of The International Marine Bivalve Workshop (IMBW), held in the Florida Keys, 19–30 July 2002, and funded by the U.S. National Science Foundation award DEB-9978119, as a part of the Partnerships in Enhancing Expertise in Taxonomy [PEET] Program, for the initial taxonomic question posed to K.F. Bennett and R. Willan while participants in the IMBW. Richard Willan was valuable at the initiation of this project. A Conchologists of America Grant to Malacology awarded to K. F. Bennett made the molecular aspects of this project possible. Thanks to the Florida Keys Marine Laboratory on Long Key for the use of facilities during the IMBW and during subsequent collecting efforts, and to Nina Bennett for assisting with specimen collection. The manuscript was greatly improved by the comments of D. Katherine Coykendall, Judith Grassle, and from two anonymous reviewers.

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